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ECBC-TR-578

EVALUATION OF TOXTRAK™ FOR ANALYSIS OF PROTEIN TOXIN TOXICITY

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October 2007

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-10-2007	Final	Jul 2005 – Jan 2006

4. TITLE AND SUBTITLE

Evaluation of ToxTrak™ for Analysis of Protein Toxin Toxicity

5a. CONTRACT NUMBER
DAAD 13-03-D-0017

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Schenning, Amanda M. (SAIC); Bevilacqua, Vicky L. H. (ECBC); Morrissey, Kevin M. (SAIC); Jeffrey S. Rice (ECBC)

5d. PROJECT NUMBER
None

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
DIR, ECBC, ATTN: AMSRD-ECB-RT-CM, APG, MD 21010-5424
SAIC, 3465A Box Hill Corporate Drive, Abingdon, MD 21009

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-578

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Ascertaining the toxicity of samples containing proteins such as ricin and SEB requires an activity assay that yields accurate and reproducible results. The ToxTrak™ system was investigated as a possible quantitative assay. ToxTrak™ is a commercially available kit supplied by the Hach Company (Loveland, Colorado). The ToxTrak™ system correlates the toxicity of a sample with its effect on the respiration of bacteria, as measured by percent inhibition. Initially, our intent was to develop a modified version of the ToxTrak™ test that would be amenable for use with a microplate reader. Development of a plate reader version of the test, however, first required the successful demonstration of the unmodified ToxTrak™ method to indicate toxicity due to protein toxins. Toxtrak™ proved to be useful for identifying the toxicity of various levels of cyanide. However, when we evaluated the kit for the ability to indicate toxicity due to a protein known to be toxic to *E. coli*, we were not able to achieve reproducible results. Due to the inconsistencies obtained for percent inhibition while utilizing the standard protocol with protein toxins, we chose not to pursue attempts to develop a modified method for use with a microplate reader.

15. SUBJECT TERMS
ToxTrak™ Cell toxicity assay Ricin Biochemical activity Activity assay

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Sandra J. Johnson
U	U	U	UL	16	19b. TELEPHONE NUMBER *(include area code)* (410) 436-2914

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PREFACE

The work described in this report was authorized under Contract No. DAAD13-03-D-0017. This work was started in July 2005 and completed in January 2006.

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Acknowledgments

The authors thank the Defense Threat Reduction Agency for funding (Project BA05TAS080, Biological Toxin Fate in Water Matrices); Dr. Stephen R. Channel for program management; Drs. H. Dupont Durst, Frederick J. Berg, David E. Tevault, and Wade D. Kuhlman for administrative support; and Janna Madren-Whalley for her technical review of the manuscript.

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EVALUATION OF TOXTRAK™ FOR ANALYSIS OF PROTEIN TOXIN TOXICITY

1. INTRODUCTION

Evaluating the toxicity of certain proteins, such as ricin and SEB, as a function of their biochemical activity in addition to their mere presence (*i.e.* by molecular mass) requires a biochemical or cell toxicity activity assay that yields accurate and reproducible results in a timely and economical manner

In this study, we investigated the ToxTrak™ system, which is a commercially available kit supplied by the Hach Company (Loveland, Colorado) as one possible assay. The ToxTrak™ system correlates the toxicity of a sample with its effect on the respiration of bacteria, as measured by percent inhibition. The system is a colorimetric test based on resazurin dye chemistry.[1] Toxicity is indicated by either the inhibition or the acceleration of the reduction of resazurin dye. This reduction is caused by the respiration of bacteria, which are added to the samples and a control. Reduction is indicated by a color change from blue to pink and a change in the value of absorbance measurements obtained with a spectrophotometer. The amount of inhibition (as percent inhibition, %I) related to the toxic sample is calculated according to the following equation:

$$\%I = [1-(\Delta A_{sample}/\Delta A_{control})] \times 100 \quad (1)$$

where ΔA = initial absorbance – final absorbance.

Percent inhibition is calculated as a function of the change of absorbance in the control sample. Because some toxins actually increase the rate of respiration of the bacteria resulting in a negative percent inhibition, those values that are either more negative than -10% or more positive than 10% are indicative of toxicity of the sample.

2. EXPERIMENTAL PROCEDURES

2.1 Materials.

The ToxTrak™ kit was obtained from the Hach Company (Loveland, CO). The kit contained all reagents necessary for sample analysis with the exception of the dehydrated *Escherichia coli* (*E. coli*). The dehydrated *E. coli* were obtained from MicroBiologics (St. Cloud, MN) in the form of KWIK-STIK swabs. Ricin (*ricinus communis agglutinin II*, RCA II, RCA_{60}) in solution (5 mg/mL) was obtained from Vector Laboratories (Burlingame, CA, product L-1090). Reagent grade sodium cyanide at >97% purity was obtained from Sigma-Aldrich (St. Louis, MO) and lysozyme from chicken egg white was obtained from Aldrich at ~95% purity.

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[1] James, R.; Dindal, A.; Willenberg, Z.; Riggs, K. *Environmental Technology Verification Report, Hach Company ToxTrak™ Rapid Toxicity Testing System*; Batelle: Columbus, OH, **2003**.

2.2 Ricin Dialysis.

Ricin as received from the vendor was dialyzed into 10 mM phosphate buffered saline (10 mM PO_4, 150 mM NaCl, pH 7.8) in preparation for ToxTrak™ studies. Dialysis was carried out employing a regenerated cellulose DispoDialyzer® (Spectrum Laboratories, Rancho Dominguez, CA) having a molecular weight cut-off of 8,000. The ricin was dialyzed over approximately 36 hr using three buffer volumes (500-650 mL each for a total of 1800 mL).

2.3 ToxTrak™ Studies.

The following is a summary of the various experiments employed during the evaluation of the Toxtrak™ kit. Except where noted, sample analysis was performed according to strict adherence to the steps provided in the ToxTrak™ Toxicity Method 10017[2] available for download on the Hach Company website at www.hach.com. Absorbance measurements were made using the Hach DR/2400 portable spectrophotometer.

Analysis was performed by transferring the contents of one ToxTrak™ Reagent Powder Pillow, 5 mL of sample and two drops of Accelerator Solution to a sample cell, capping and shaking to mix. A control sample was prepared in the same manner using 5 mL of de-ionized water as the sample matrix. The prepared *E. coli* inoculum (0.5 mL) was added to each sample cel, and the cells were inverted to mix. The control sample was immediately placed in the spectrophotometer, and an initial absorbance measurement was obtained at 603 nm. Following the control sample, initial absorbance measurements were obtained for each sample. The solutions were allowed to react until the absorbance of the control sample decreased 0.60 ± 0.10 absorbance units. After the absorbance of the control sample decreased 0.060 ± 0.10 absorbance units and was recorded, the absorbances of the remaining samples were immediately obtained and recorded.

It should be noted that at the time of testing, the vendor had recently discontinued providing the AQUA QC-Stik™ as part of the test kit but had not yet updated the inoculum preparation procedure in Method 10017. At the recommendation of the vendor, dehydrated *E. coli* was obtained from MicroBiologics, (St. Cloud, MN) in the form of KWIK-STIK swabs. The KWIK-STIK swabs were activated according to the enclosed instructions and then immersed in either Total Bacteria Count Broth (Bacteria Count) Tubes or Laurel Tryptose Broth (Laurel Tryptose) Tubes provided with the ToxTrak™ kit. The tubes were then incubated at 35 °C for 12-18 hr until the contents were visibly turbid, indicating bacterial growth. New tubes were inoculated by inverting the original culture tube and switching caps with the new tube. From this point on, tubes inoculated directly from swabs are referred to as parent cells, and tubes inoculated by contact with the caps from parent cells are referred to as daughter cells.

[2] ToxTrak™ Method (0 to 100% Inhibition), DR/2400 Toxicity Method 10017. www.hach.com (accessed October 2007).

3. RESULTS AND DISCUSSION

3.1 Experimental Design.

Initially, the intent in working with the ToxTrak™ system was to develop a modified version of the test that would be amenable to use with a microplate reader. To minimize the hazards associated with manipulating toxic proteins such as ricin and SEB in the laboratory, as well as to offset the expense of these materials, one goal was to keep the required sample volumes to a bare minimum. Volumes in the range of 100 to 300 μL would allow a wide range of protein concentrations, while using a minimum amount of toxic material. The use of a microplate reader to measure the absorbance during toxicity testing would facilitate this goal. Development of a plate reader version of the test, however, relied strictly upon the successful demonstration of the unmodified ToxTrak™ method to indicate toxicity due to protein toxins. The experimental plan was to first test the standard method on a variety of sample matrices of known toxicity and then to adapt the method for use with the plate reader.

3.2 Method Evaluation with Non-Protein Toxic Matrices.

To assess the potential use of the ToxTrak™ system as an indicator of biochemical activity in protein toxins, its ability to indicate toxicity due to the presence of a non-protein toxin such as cyanide was first verified.

The sample matrix was prepared by dissolving sodium cyanide in de-ionized water and serially diluting to obtain four sample solutions containing CN^- at concentrations ranging from 0.250 to 250 mg/L. Each of the four samples was analyzed according to the ToxTrak™ method, using parent cell inoculum prepared in a Bacteria Count tube. Table 1 details the percent inhibition calculated for each solution.

Table 1. Cyanide Percent Inhibition for Single Samples

Sample	Initial Absorbance	Final Absorbance	Delta Absorbance	Inhibition (%)	Toxicity
Control	1.616	1.075	0.541	0.00	
0.25 mg/L	1.513	1.018	0.495	8.50	-
2.5 mg/L	1.556	1.036	0.520	3.88	-
25.0 mg/L	1.492	1.028	0.464	14.23	+
250 mg/L	1.347	1.186	0.161	70.24	+

The outcome of this preliminary experiment with cyanide suggested that, if the ToxTrak™ method could be shown to yield consistent and reproducible results for cyanide, it may prove to be a reliable indicator of sample toxicity for protein toxins as well.

To assess the reproducibility of the test results obtained with aqueous cyanide, the test was repeated four more times, using two different sources of parent cell *E. coli* inoculum prepared in Laurel Tryptose tubes. Four sample sets of aqueous cyanide, in concentrations that ranged from 0.25 to 500 μg/mL, were prepared individually by serial dilution from one cyanide stock solution. The results of these studies are summarized in Table 2 and demonstrate that the ToxTrak™ kit consistently indicated the presence of toxic levels of cyanide in an aqueous sample matrix.

Table 2. Cyanide Percent Inhibition Reproducibility

Concentration (μg/L)	Inhibition for Each of Four Samples (%)	Average Inhibition (%)	Standard Deviation (%)	Toxicity
0.25	3 -15 0 20	2	14	-
2.5	9 5 26 33	18	14	+
25	11 26 33 21	23	9	+
100	23 55 38 31	37	14	+
250	53 65 80 58	64	12	+
500	89 89 92 92	90	2	+

3.3 Method Evaluation with Protein Toxins.

3.3.1 Lysozyme Sample Matrix.

Initial investigations into the ability of the ToxTrak™ kit to detect the activity of bio-toxins were carried out using the enzyme lysozyme. In addition to its widespread availability and relative low cost, lysozyme is well known for its antibiotic properties and presents far less hazard in the laboratory as compared to other protein toxins. Based upon these factors, lysozyme was chosen as a favorable protein to act as a surrogate for ricin and SEB in the initial stages of experimentation.

Several trials were initially performed with lysozyme at various concentrations ranging from 0.01 to 122 μg/mL. Sodium azide was included as a preservative in the lysozyme samples at the same concentration as in the ricin from the vendor (Vector Laboratories). These results were inconsistent. Two experiments were then carried out without sodium azide and also included some higher lysozyme concentrations.

Table 3 compares all the initial lysozyme experiments (with and without sodium azide), revealing that concentrations at or below 25 μg/mL gave inconsistent results, while concentrations of 50 μg/mL or greater produced consistently positive results. However, even at lysozyme concentrations above 50 μg/mL, the percent inhibition values were not very reproducible and, therefore, not suitable for a quantitative assay. During these studies, some tests were performed using parent cells, while some tests utilized daughter cells as the instructions allowed for the use of either. Furthermore, the inoculum, whether parent or daughter, was inadvertently grown in Bacteria Count tubes as opposed to Laurel Tryptose tubes. In an attempt to minimize possible sources of variation and achieve more reproducible results, two additional experiments were performed using lysozyme that was not preserved by sodium azide. A comparison study was conducted, using only parent cell inoculum that was grown side by side in Bacteria Count tubes and in Laurel Tryptose tubes. The entire experiment was repeated the following day. The lysozyme concentrations in the samples ranged from 0.01 to 500 μg/mL. The results of these experiments are summarized in Table 4 and Figures 1 and 2.

These tests resulted in substantial inconsistencies in the prediction of toxicity of the lysozyme samples. Significantly different values were obtained for percent inhibition at most concentration levels, even above 50 μg/mL, frequently yielding conflicting indications as to toxicity of the sample. These variations occurred regardless of the inoculum used during testing. Results of this study suggest that the ToxTrak™ kit may not be applicable to all protein toxins and that each toxin may need to be evaluated individually.

Table 3. Initial Trials with Lysozyme Using Inoculum Grown in Bacteria Count Tubes

Lysozyme Concentration (μg/mL)	Trial 1 (parent cells)		Trial 2 (daughter cells)		Trial 3 (daughter cells)		Trial 4 (daughter cells)		Trial 4 (parent cells)	
	Inhibition (%)	Toxic (+/-)	Inhibition (%)	Toxic (+/-)	Inhibition (%)	Toxic (+/-)	Inhibition (%)	Toxic (+/-)	Inhibition (%)	Toxic (+/-)
0.01	-7	-							-17	+
0.1	3	-	-33	+					-8	-
1	7	-	-10	+					-14	+
2.5							-2	-	4	-
10-15	1	-	1	-					11	+
25			6	-			11	+	16	+
50									21	+
125					12	+	13	+	33	+
250							31	+	33	+
500							60	+	60	+

Table 4. Comparison Study with Lysozyme Using Inoculum Grown in Laurel Tryptose Tubes versus Bacteria Count Tubes

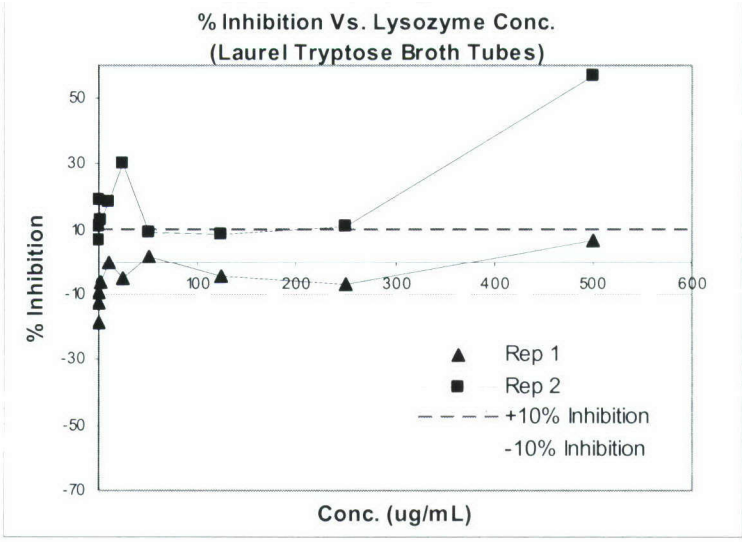
Inoculum	Lysozyme Concentration (μg/mL)	Rep 1 Inhibition (%)	+/-	Rep 2 Inhibition (%)	+/-
Laurel Tryptose	0.01	-10	+	11	+
	0.1	-18	+	7	-
	1	-12	+	19	+
	2.5	-6	-	13	+
	10	0	-	19	+
	25	-5	-	30	+
	50	2	-	9	-
	125	-4	-	9	-
	250	-7	-	11	+
	500	7	-	57	+
Bacteria Count	0.01	-49	+	4	-
	0.1	-45	+	3	-
	1	-51	+	22	+
	2.5	-36	+	-5	-
	10	-61	+	9	-
	25	-56	+	9	-
	50	-50	+	7	-
	125	-28	+	15	+
	250	-7	-	17	+
	500	40	+	49	+

3.3.2 Ricin Sample Matrix.

A final study was conducted to evaluate the ToxTrak™ kit with aqueous samples containing varying levels of ricin. Ricin is a protein toxin comprised of an A and B chain linked together by a disulfide bond. With the A and B chains intact, ricin is able to transgress the membrane of a eukaryotic cell and inhibit its ability to produce proteins necessary to sustain life, eventually causing the cell to die. Athough ToxTrak™ employs prokaryotic cells (*E. coli*), it has been reported to be a useful indicator of ricin toxicity.[1]

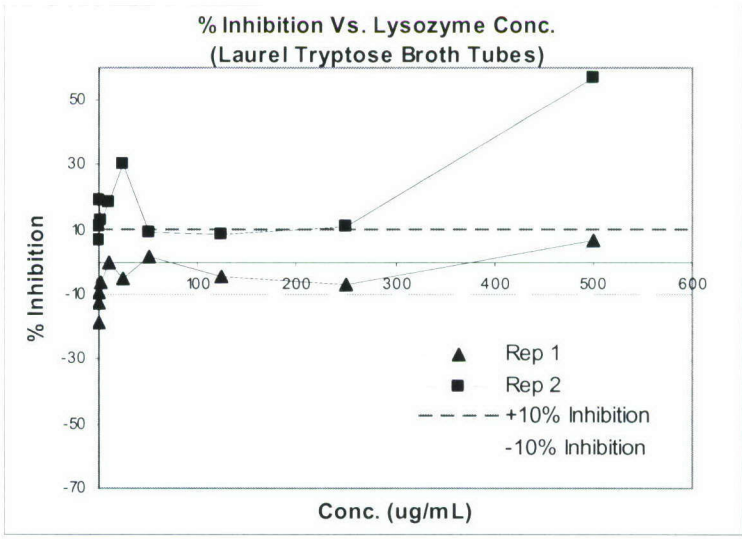
For this study, intact ricin was used to prepare samples of varying concentrations to be tested with parent *E. coli* cell inoculum grown in Laurel Tryptose tubes. Prior to use, the ricin stock solution was dialyzed into phosphate buffer to remove any traces of the sodium azide preservative. Assuming no change in concentration of the ricin stock solution following dialysis, the ricin content of the prepared samples ranged from 0.015 to 60 μg/mL. The toxicity test was performed on each sample in duplicate. Results of this study are outlined in Table 5 and illustrated in Figure 3. Again, the results were inconsistent.

[1]James, R.; Dindal, A.; Willenberg, Z.; Riggs, K. *Environmental Technology Verification Report, Hach Company ToxTrak™ Rapid Toxicity Testing System*; Batelle: Columbus, OH, **2003**.



▲ = Replicate 1
■ = Replicate 2

Figure 1. Toxicity Testing Performed on Two Sets of Lysozyme Samples on Two Consecutive Days, where the Inoculum was Grown in Laurel Tryptose Tubes.

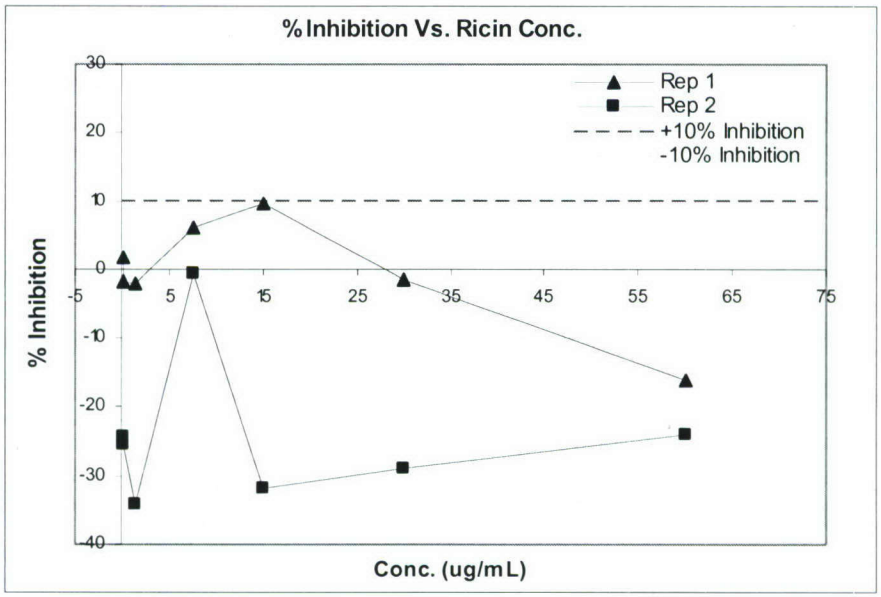


▲ = Replicate 1
■ = Replicate 2

Figure 2. Toxicity Testing Performed on the Same Two Sets of Lysozyme Samples as Used for Figure 1 on Two Consecutive Days, where the Inoculum was Grown in Bacteria Count Tubes.

Table 5. Toxicity Testing of Aqueous Ricin Samples

Ricin Concentration (μg/mL)	Rep 1 Inhibition (%)	+/-	Rep 2 Inhibition (%)	+/-
0.015	2	-	-24	+
0.15	-2	-	-26	+
1.5	-2	-	-34	+
7.5	6	-	-1	-
15	10	+	-32	+
30	-2	-	-29	+
60	-16	+	-24	+



▲ = Replicate 1
■ = Replicate 2

Figure 3. Percent Inhibition versus Ricin Concentration.

4. CONCLUSIONS

The Toxtrak™ proves to be a useful tool for identifying the toxicity of various levels of cyanide in samples. However, when we evaluated the kit for the ability to indicate toxicity due to a protein known to be toxic to *E. coli*, we were not able to achieve reproducible results. Due to the inconsistencies obtained for percent inhibition while utilizing the standard protocol with protein toxins, we chose not to pursue attempts to develop a modified method for use with a microplate reader.

The conclusions drawn in this study appear consistent with the test results described in the "Environmental Technology Verification Report" prepared by Battelle of Columbus, Ohio.[1] This report concluded that "The contaminants that were analyzed by ToxTrak™ during this verification test produced results with a high degree of variability, making it difficult to quantitatively interpret the data. The only contaminant that met the requirements for quantitative detection … was cyanide."[1]

[1]James, R.; Dindal, A.; Willenberg, Z.; Riggs, K. *Environmental Technology Verification Report, Hach Company ToxTrak™ Rapid Toxicity Testing System*; Batelle: Columbus, OH, **2003**.